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Our laboratory is studying anastellin, a fragment of fibronectin that inhibits angiogenesis, tumor growth and metastasis *in vivo*. By fusing anastellin with peptides that home to specific vascular sites in angiogenic vasculature, I am testing whether the concentration of anastellin at the angiogenic endothelium can be increased, and whether that would improve the anti-angiogenic activity of anastellin. I have found that targeting anastellin to angiogenic blood vessel endothelium *in vivo* does not improve the efficacy of anastellin. However, in contrast, targeting anastellin to lymphangiogenic vessels has a significant inhibiting effect on the development of lymphatic vasculature. These findings are interesting, because eliminating tumor lymphatics is a key step in inhibiting tumor metastasis.

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INTRODUCTION

Our laboratory has developed a substance, anastellin, a fragment of fibronectin that inhibits angiogenesis, tumor growth and metastasis *in vivo* (*1-3*). By using *in vivo* screening of phage libraries (*4*), our laboratory has also isolated peptides that specifically home to the vasculature of tumors (*5*), in particular to human breast cancer xenografts (*6, 7*). My project aims at combining these two tools into one cancer therapeutic, and to test the efficacy of this combination against breast cancer in mice. The goal of this project is thus to target anastellin to specific vascular sites *in vivo*, by fusing anastellin with homing peptides that home to the vasculature of breast cancers. My hypothesis is that, by targeting anastellin to the tumor vasculature, the concentration of anastellin at the angiogenic endothelium will increase, improving the efficacy of anastellin.

BODY

The approved specific aims of this project are:

(i) To design variants of anastellin with a homing peptide at either end of the poly-peptide in order to define lead compounds.

(ii) To test these compounds *in vivo*.

Our long-term goal is to create an efficient anti-tumor compound specifically designed for treatment of breast cancer. During this first year of the project, I have made significant progress with both aims (i) and (ii).

I designed three different targeted variants of anastellin. Three homing peptides, "F3", "LyP1", and "RGD4C" were fused to anastellin. RGD4C binds specifically to the a_vb_3 and the a_vb_5 integrins (*8, 9*), F3 binds to blood vessels and tumor cells in various tumors (*6*), and LyP1 recognizes lymphatic vessels and tumor cells in certain tumors (*7*).

To find the optimal design of peptide-fused anastellin, we expressed different variants on the surface of phage and tested for binding to tumor cells *ex vivo*, and homing to tumor *in vivo*. The LyP1 peptide was placed either at the N-, or the C-terminus, or at both ends. I found that the best results were obtained with the peptide at the N-terminus of anastellin, and with a three-glycine linker in between (Figure 1). Thus, the other peptides, F3 and RGD4C, were also positioned at the N-terminus of anastellin with a three-glycine linker.

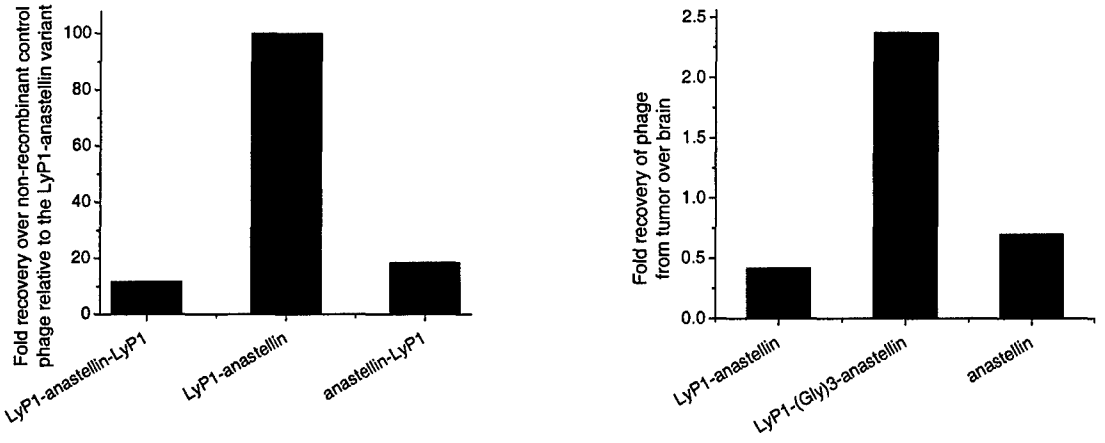

Figure 1. The binding to tumor cells from subcutaneous MDA-MB-435 tumors of phage expressing different variants of LyP1 fused with anastellin was tested *ex vivo*. The best binding was obtained with LyP1 fused to the N-terminus of anastellin (**left**). Two variants of LyP1-fused anastellin, one with and one without a three-glycine linker between peptide and anastellin, were tested for homing to subcutaneous breast tumors *in vivo*. The variant with the linker was found to home the best (**right**).

The peptide-fused anastellin variants were tested for anti-angiogenicity using matrigel plugs. Although this experiment was not described in the original application, it is a fast and easy way to test for anti-angiogenic activity. Neither F3- nor RGD4C-fused anastellin had any effects on the development of blood vessels in the plugs (data not shown). Hence, I have not pursued further work with those variants. In contrast, I found a clear anti-lymphangiogenic effect with LyP1-anastellin (Figure 2).

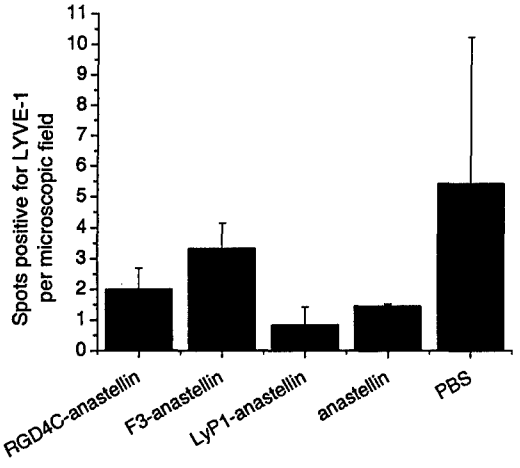

Figure 2. Matrigel plugs containing bFGF were implanted into mice, which were treated with daily intravenous injections of 800 ug protein or vehicle for 7 days, starting the day after the implantation. The plugs were removed on day 8 and the density of cells positive for the lymphatic marker LYVE-1 was quantified under the microscope at 400X magnification.

Subcutaneous human breast carcinomas were transplanted into the chest area of nude mice, and were allowed to grow for 4.5 weeks until the tumors were clearly visible and palpable. After treatment with LyP1-anastellin twice per week for 5 weeks, the density of lymphatic vessels was drastically reduced (Figure 3). This experiment has been repeated twice, both times with similar results.

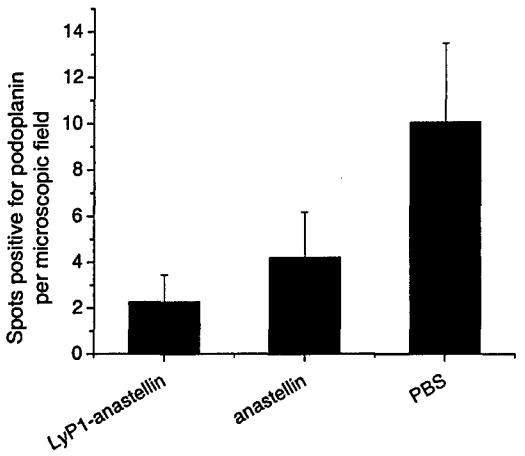

Figure 3. Human breast carcinoma cells MDA-MB-435 were implanted into mice as allowed to grow for 4.5 weeks until the tumors were palpable. The mice were treated twice per week with intravenous injections of 800 ug protein or vehicle for 4.5 weeks. The tumor was then removed and sectioned, and the density of cells and vessels positive for the lymphatic marker podoplanin was quantified under the microscope at 400X magnification.

These results show that anastellin indeed can be targeted, although not to angiogenic blood vessels. Anastellin may need to be in circulation to be an active angiogenesis inhibitor. However, targeting the tumor lymphatic vessels and inhibiting lymphangiogenesis opens up the possibility to inhibit the spreading of circulating tumor cells. We are currently exploring the full potential of LyP1-anastellin in an *in vivo* metastasis study. We also plan to combine LyP1-anastellin, to eliminate the development of lymphatic vessels, with an efficient blood vessel inhibitor. This combination could result in synergistic effects.

KEY RESEARCH ACCOMPLISHMENTS

- The homing of anastellin targeted with a tumor lymphatic homing peptide, LyP1, was tested by using *in vivo* phage display. The optimal position of the homing peptides was shown to be at the N-terminus of anastellin.

- Three targeted variants of anastellin and control fragment have been cloned and expressed in bacteria cultures. The targeted variants are: LyP1-, F3- and RGD4C-anastellin.

- None of the variants are active in inhibiting angiogenesis in matrigel plug assays *in vivo*.

- One variant, LyP1-anastellin, is efficient in inhibiting lymphangiogenesis when tested in matrigel plug assays, and in tumor treatment studies.

REPORTABLE OUTCOMES

None yet.

CONCLUSIONS

I have made significant progress toward understanding how anastellin best can be targeted to vascular sites *in vivo*. Although anastellin is thought to act on blood vessel endothelial cells, targeting anastellin to those sites either inactivates anastellin, or inhibits it from acting at the site of angiogenesis.

In contrast, targeting anastellin to tumor lymphatics has a significant inhibition effect on the development of tumor lymphatics. The ability to inhibit tumor lymphangiogenesis may be very important for limiting the spreading of lymphatic-bourne tumor metastasis. We have thus defined LyP1-anastellin as our lead compound for further studies.

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